

REMARKS

Claims 1, 5, 6, 16, 20 and 21 are all the claims pending in the application.

Claims 1, 5, 6, 16, 20 and 21 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Brantman (US 4,687,782) in view of Soop *et al.* (1988 J Appl Physiol 64(6): 2394-2399).

This rejection should be withdrawn for at least the reasons set forth in the Remarks section of the Response under 37 C.F.R. § 1.116 filed September 8, 2010 and for the additional reasons set forth below.

In the Response under 37 C.F.R. § 1.116 filed September 8, 2010, Applicants explained that (1) the legal precedent cited by the Examiner does not apply to the present case because Brantman specifically requires the use of carnitine in his composition for its intended purpose; (2) although Soop *et al.* teach that adequate muscle carnitine levels are maintained during exercise and that carnitine supplementation has no substantial effect on skeletal muscle metabolism under normal physiological conditions, Soop *et al.*'s study relates to the single use of carnitine and its effect on skeletal muscle metabolism under normal physiological conditions. That is, the level of carnitine taught by Soop *et al.* might not be adequate for the purpose taught by Brantman; (3) the composition defined in the present claims is patentable over Brantman from the fact that the claimed composition retains and improves the desired function even canitine, which was an essential element of Brantman's composition, is omitted; (4) one of ordinary skill would recognize that according to Brantman, omission or exclusion of carnitine would markedly reduce desired physiological effects of the composition; accordingly, one of ordinary skill in the art would not have prioritized the cost reduction effects at the cost of such physiological effects.

In response, the Examiner states:

(1) Applicants are reminded that the 'subject matter as a whole' which should always be considered in determining the obviousness of an invention under 35 U.S.C. § 103. Brantman discloses that carnitine is employed to optimize skeletal muscle function in relation to oxidation of fatty acids for calories (col. 4 lines 8-13); however, Brantman also discloses that carnitine is synthesized in the body (col. 3 lines 59-60) and that it has the smallest amount in the composition (col. 4 lines 15-50). Therefore, since it was known in the art at the time of the invention that adequate muscle carnitine levels are maintained during exercise and that carnitine supplementation has no substantial effect on skeletal muscle metabolism under normal physiological conditions (Soop et al.), it would be reasonable for one of ordinary skill to exclude carnitine from the composition of Brantman since exogenous carnitine does not appear to have a substantial effect on skeletal muscle. Additionally, it would be reasonable for one of ordinary skill to know that excluding carnitine would save time and money because it is not absolutely required since there is endogenous carnitine present in the body.

(2) Soop et al. disclose that adequate muscle carnitine levels are maintained during exercise, therefore, there would still be an appropriate level of carnitine present in the body when said composition of Brantman, without carnitine present, is administered during exercise, such that the branched amino acids will be oxidized and toxic ammonia will be removed.

(3) The exclusion of carnitine from the composition of Brantman would still yield a composition that has the same function and properties as the instant [Brantman's] composition which includes carnitine because, endogenous carnitine is produced in the body and maintained at an adequate level during exercise (Brantman, Soop et al.).

(4) Both Brantman and Soop *et al.* disclose that endogenous carnitine is produced in the body and Soop *et al.* further disclose that carnitine is maintained at an adequate level during exercise.

Applicants respectfully disagree.

Brantman clearly teaches, at col. 3, line 55 to col. 4, line 30, the use of carnitine in his composition and the object of the use of carnitine is to optimize skeletal muscle function in relation to oxidation of fatty acids for calories; to the oxidation of branched amino acids (BAAs); and to enhance the removal of toxic ammonia.

Further, Brantman teaches that the objects of the invention are realized by a careful selection of specific amino acids to be added to whole protein and other nutrients, so as to achieve a diet which is enriched with specific amino acids (carnitine, glutamine, isoleucine, leucine and valine), in order to maximize protein synthesis in skeletal muscle (col. 4, lines 25-30).

Although Soop *et al.* teaches that carnitine supplementation has no substantial effect on skeletal muscle metabolism under normal physiological conditions, Soop *et al.*'s study relates to the single use of carnitine and its effect on skeletal muscle metabolism under normal physiological conditions.

In contrast, Brantman teaches that "This invention relates to compositions of specific nutrients to facilitate the adaption [adaptation] of skeletal muscle to programs of strenuous exercise."

Thus, Soop *et al.* is not sufficient to provide motivation to one skilled in the art to omit carnitine from the composition of Brantman, because omission of carnitine from the composition of Brantman would render it unsatisfactory for its intended purpose; relevant law holds that if a

proposed modification would render a prior art invention being modified unsatisfactory for its intended purpose, then there is no suggestion or motivation to make the proposed modification.

In re Gordon, 733 F.2d 900, 221 USPQ 1125 (Fed. Cir. 1984).

Further, Applicants submit herewith three publications which were published after Brantman (1987) and Soop et al. (1988) but before 2003, when the instant application was filed, which report the decreases in carnitine level in muscle in athlete during 1 - 6 months training and the importance of a carnitine supplement to prevent such decrease.

- Arenas J. et al., "Carnitine in muscle, serum, and urine of nonprofessional athletes: effects of physical exercise, training, and L-carnitine administration," Muscle Nerve 1991, 14, 598-604;
- Huertas R. et al., "Respiratory chain enzymes in muscle of endurance athletes: effect of L-carnitine," Biochem. Biophys. Res. Commun., 1992, 188, 102-107; and
- Arenas J., et al., "Effects of L-carnitine on the pyruvate dehydrogenase complex and carnitine palmitoyl transferase activities in muscle of endurance athletes," FEBS Lett., 1994, 341, 91-93.

Applicants also submit a Declaration under 37 C.F.R. § 1.132, in which Mr. Masayuki Suzuki reviews and discusses each publication.

As Mr. Suzuki states in the Declaration, Arenas (1991) teaches that carnitine amount in muscle decreases in athlete during 1 to 6 months training and that decrease in amount of carnitine in muscle can be prevented according to training by supplementation of carnitine. Huertas and Arenas (1994) teach that activities of enzymes in muscle which become key enzymes (such as puruvate dehydrogenase and electron transport chain enzymes) can be increased.

According to Mr. Suzuki, these publications show that carnitine is not maintained at an adequate level during exercise, contrary to the teaching of Soop et al.

Mr. Suzuki further states that one skilled in the art would not have been motivated to remove carnitine from Brantman composition, when reading the teachings of Brantman, Soop, and the Arenas (1991), Huertas, and Arenas (1994), because Arenas (1991), Huertas, and Arenas (1994), in addition to Brantman, clearly emphasize the function of carnitine supplement for an athlete who is undertaking strenuous exercise for a long term (e.g., 1-6 months).

Thus, the Examiner's finding and reliance of Soop et al. are based on improper hindsight only after reading the specification of the present application. As disclosed by Brantman, there are reports which disclose that carnitine is important for oxidation of fatty acids, removal of ammonia and oxidation of BAAs during exercise. Among the findings which are available for the person ordinary skilled in the art, the Examiner intentionally selected only Soop et al. However, the person ordinary skilled in the art is a person who can access all publications and it can be assumed that the person ordinary skilled in the art also knew references that contradict Soop et al.

The Examiner states that removal of carnitine from the composition of Brantman is obvious based on the teaching of Soop et al. This is a standard "obvious to try" and whether the rejection is appropriate is decided by whether there was a "reasonable expectation of success" for the person ordinary skilled in the art. While there was Soop et al., there were also publications which reported importance of carnitine during exercise as taught by Brantman and by, for example, the three publication discussed above. Therefore, it cannot be reasonably expected that the object of Brantman can be successfully achieved when carnitine is removed from the

composition of Brantman. If information in both positions was available, it cannot be said that there was reasonable expectation of success.

Further, as discussed above, Brantman clearly teaches that carnitine is a critical component of the composition. Therefore, Brantman teaches away from the present invention where carnitine is excluded. A claimed combination of prior art elements may be nonobvious where the prior art teaches away from the claimed combination and the combination yields more than predictable results. See, *Crocs, Inc. v. U.S. Int'l Trade Comm'n*, 598 F.3d 1294 (Fed. Cir., 2010).

In view of the above, the present claims are not obvious and are patentable over Brantman and Soop *et al.*, either alone or in combination. Reconsideration and withdrawal of the §103(a) rejection based on Brantman in view of Soop *et al.* are respectfully requested.

Allowance is respectfully requested. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

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Efficient utilization of fatty acids to sustain prolonged physical efforts is thought to be dependent on the carnitine shuttle of muscle. A study has been carried out in 24 athletes (13 long-distance runners and 11 sprinters). These subjects received placebo or L-carnitine (1 g/orally b.i.d.) during a 6-month period of training. In endurance athletes, training induced lowering of total and free muscle carnitine. Increase of esterified muscle carnitine was also observed. Post-exertional overflow of acetylcarnitine and long-chain acylcarnitine, as well as reduction of the free fraction was also noticed in the blood. Fasting plasma carnitine levels, however, were not affected in carnitine-treated athletes at rest. These changes were likely related with the significantly increased urinary excretion of esterified and total carnitine which occurred after physical exercise. In the sprinters only, a decrease in free and total carnitine of muscle was detected after training. Both these potentially unfavorable effects were prevented by oral administration of L-carnitine. Our data suggest that training in endurance athletes, and to a lesser extent, in sprinters, is associated with a decrease in free and total carnitine of muscle, due to an increased overflow of short-chain carnitine esters in urine.

Key words: carnitine • acylcarnitine • long-distance runners • sprinters • athletes • exercise

MUSCLE & NERVE 14:598-604 1991

CARNITINE IN MUSCLE, SERUM, AND URINE OF NONPROFESSIONAL ATHLETES: EFFECTS OF PHYSICAL EXERCISE, TRAINING, AND L-CARNITINE ADMINISTRATION

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Muscle performance during aerobic exercise relies upon increasing supply, as well as improved utilization of oxidizable substrates. Breakdown of stored glycogen and triglycerides sustains physical exercise. In addition, within minutes from the onset of exercise, muscular blood flow increases, thus delivering to the muscle fibers larger amounts of

both oxygen and nutrients (i.e., glucose and free fatty acids, FFA). However, if physical effort is continued for a longer time, long-chain fatty acids (LCFA) become the main (almost the exclusive) source of energy for the working muscle.⁹

Carnitine performs a crucial role in the energy supply of muscle during exercise, by controlling the influx of fatty acids into mitochondria.⁸ Carnitine, either introduced with the diet or synthesized *de novo* in the liver and kidney, must be actively concentrated from the blood into muscle.¹⁸ Here, two enzymes, carnitine palmitoyl transferase (CPT) and carnitine acetyl transferase (CAT), embedded in the mitochondrial inner membrane, utilize carnitine as a shuttle to direct long-chain fatty acids and acetyl groups in and, respectively, out of mitochondria.^{7,13}

Additional functions of carnitine could also be important during physical exercise: carnitine facilitates oxidation of pyruvate and branched amino acids²¹ and, by preventing the accumulation of acylCoAs, contributes to the protection of cells

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from these membrane-destabilizing agents.²⁰ A complex equilibrium exists among the various carnitine fractions; i.e., free vs. acylated forms, and between the carnitine pool of muscle vs. that in the blood, as well as the fraction excreted in the urine.¹⁹ Ultimately, this equilibrium determines the size of the muscular pool of free carnitine, which, from an energetic standpoint, represents the metabolically active fraction. Therefore, the amount of free carnitine in muscle can be considered a limiting factor for its energy supply.

During exercise, especially if prolonged, the carnitine fractions of the body compartments change in size, mainly because of the increased esterification of muscle free carnitine with acyl groups from different sources.^{1,14,15} Because this phenomenon is chronically activated by physical training, a possible consequence of the latter condition is a progressive reduction of the free carnitine pool in muscle, which could lead to a potentially deleterious "carnitine insufficiency."⁴ The aim of the present study is to verify, at the biochemical level, how physical training and/or exercise modify carnitine metabolism in subjects with and without carnitine supplementation. Moreover, we have investigated whether these changes are specific for athletes performing predominantly aerobic exercises, compared with athletes performing predominantly glycolytic exercises.

METHODS

Subjects and Experimental Protocols (Fig. 1). All the subjects were volunteers and expressed their informed consent to participate in the study.

Twenty-four athletes (double blind, parallel groups) were studied. Thirteen subjects (all males, ages from 19 to 27) were long-distance runners, a specialty requiring physical endurance, while 11 subjects (6 males and 5 females, ages from 19 to 21) were sprinters, a specialty requiring brief, maximal physical efforts.

Long-distance runners had a dietary regimen of 3500 to 4000 kcal/d, with the proteins representing 13% to 15% of the total caloric intake, carbohydrates 55% to 60%, and lipids 25% to 30%.

Male sprinters had the same caloric intake but proteins were 18% to 20%, carbohydrates 55% to 60%, and lipids 20% to 25%. Female sprinters had a caloric intake of 3000 to 3500 kcal/d with the same diet as males. Blood specimens were always collected in the morning after an overnight fast. Determination of "basal" values was obtained by measuring free carnitine, acetyl carnitine, short-chain acylcarnitine, long-chain acylcarnitine and total carnitine during the pretraining period.

The same assays were also carried out in muscle needle biopsies (vastus lateralis, average wet weight: 100 to 150 mg) taken at rest, as well as in the serum and in the 24-hour urine, both collected the day before and the day after a 400-

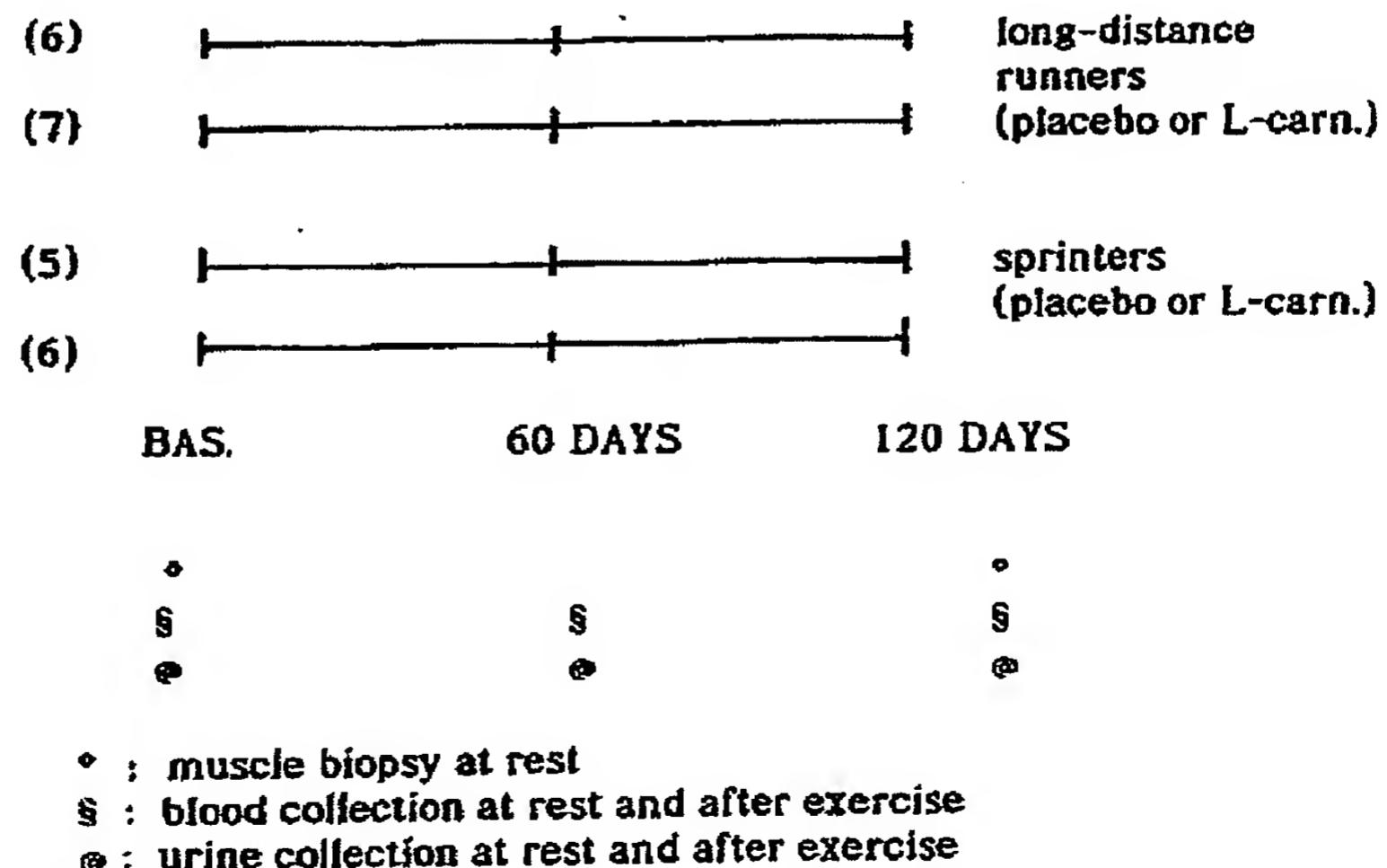


FIGURE 1. Design of the study. Two groups of athletes were sprinters (11 athletes) and two groups were long-distance runners (13 athletes).

meter and a 3000-meter run for the sprinters and for the long-distance runners, respectively.

The long-distance runners were then divided at random in two groups: the first group (7 males) was treated with 1 g orally b.i.d. of L-carnitine (Sigma Tau, Madrid, Espana) for 120 days; the other group (6 males) received placebo during the same period of time. Carnitine supplementation was suspended 12 hours before blood, urine, and muscle sampling.

Also, the sprinters were randomly assigned to the treatments: 3 males and 3 females were treated with L-carnitine and 3 males and 2 females with placebo. Dose, route of administration, and length of treatment were the same as for the long-distance runners. At the same time, the athletes of the four groups started a 4-month physical training program in their respective fields.

The weekly training program of the long-distance runners was physical exercise at 40% to 50% of the $\dot{V}O_2$ max for 1 h/d for 4 days, and at 70% to 80% of the $\dot{V}O_2$ max for 90 minutes for the other 3 days, corresponding to 70 to 80 km/wk.

The weekly training program for the sprinters was physical exercise at 40% to 50% of the $\dot{V}O_2$ max for 1 h/d for the first day, and 90 minutes of training above maximum anaerobic threshold for the other 6 days, corresponding to 25 to 30 km/wk.

Determination of carnitine levels at rest and after physical exercise was performed in serum and urine of the athletes after 60 and 120 days of training. At the end of the training period (120 days), a second biopsy was also performed (at rest) and carnitine content, as well as fiber size and morphometric evaluation, were re-examined.

Muscle Morphology. A fragment of each needle biopsy was immediately frozen in isopentane cooled in liquid nitrogen for morphological studies, including histoenzymatic reactions for alkaline and acid-sensitive ATPase, NADH-tetrazolium reductase, and succinate dehydrogenase.² Lipid and polysaccharide content and distribution were evaluated by oil-red O and PAS reactions.² The morphometric studies consisted of measuring the number and size of the fibers according to types and subtypes.⁸ Fibers were measured on a TV monitor-digitalized picture taken from microscope by a video camera. Measurement was manually performed by marking the limit of the fibers with a "cursor pen" on a "digitizer tablet." Units were expressed in microns.

Carnitine Assay. Free carnitine (FC), acetylcarnitine (AC), short-chain acylcarnitine (SCAC), long-chain acylcarnitine (LCAC), and total carnitine (TC) were measured in muscle homogenates, serum, and urine as described by DiDonato et al⁵ (FC, SCAC, LCAC, and TC) and Pande et al (AC).¹⁷

Statistical Analysis. Statistical analysis was performed by two-way analysis of variance and Student's *t* test for paired and unpaired comparison. Analysis of variance was applied to point out the difference between the two groups of athletes (long-distance runners and sprinters) while the test was used for the pairwise comparison of the values recorded at the baseline and after 60 and 120 days of treatment. Values in the tables are expressed as means \pm SD. Only differences with $P < 0.05$ were considered statistically significant.

RESULTS

Muscle (Table 1). In basal conditions, muscle free and total carnitine contents were both significantly ($P < 0.01$) higher in the long-distance runners compared with the sprinters.

Training significantly decreased the amounts of both TC ($P < 0.01$) and FC ($P < 0.02$) in the long-distance runners receiving placebo compared with the pretraining levels, whereas SCAC was unchanged, and LCAC was slightly increased. By contrast, carnitine and its esters were significantly higher ($P < 0.05$ to $P < 0.01$, Table 1) after training compared with the pretraining period in the athletes treated with oral carnitine. In addition, the difference between carnitine-treated athletes and placebo-receiving athletes after training was highly significant for free carnitine ($P < 0.001$) and total carnitine ($P < 0.001$).

Similarly, in the sprinters receiving placebo, training significantly decreased FC and TC levels, while they were increased after L-carnitine supplementation. Again, the differences between the carnitine-treated group and the placebo group after training were significant for both free carnitine ($P < 0.001$) and total carnitine ($P < 0.001$).

Serum (Table 2). In contrast to the results observed in muscle, the fasting levels of free, esterified, and total carnitine in the serum, measured at rest in both groups of athletes, were not modified by either training, carnitine supplementation, or both. However, after physical exercise, the long-distance runners receiving placebo showed a post-

Table 1. Muscle free carnitine (FC), acetylcarnitine (AC), short-chain acylcarnitine (SCAC), long-chain acylcarnitine (LCAC), and total carnitine (TC) in 24 athletes (13 long-distance runners and 11 sprinters).

	Placebo		L-Carnitine	
	Before training	After training	Before training	After training
<i>Muscle: Long-distance runners</i>				
FC	24.7 ± 1.8	22.9 ± 1.8	23.4 ± 3.9	25.7 ± 2.6 (<i>P</i> < 0.01)
AC	2.6 ± 0.4	2.6 ± 0.2	2.3 ± 0.6	2.6 ± 0.5 (<i>P</i> < 0.05)
SCAC	2.8 ± 0.4	2.7 ± 0.4	2.5 ± 0.6	2.8 ± 0.5 (<i>P</i> < 0.05)
LCAC	0.40 ± 0.06	0.48 ± 0.07	0.33 ± 0.11	0.47 ± 0.08 (<i>P</i> < 0.02)
TC	28.0 ± 2.0	26.3 ± 1.9 (<i>P</i> < 0.01)	26.3 ± 4.1	29.0 ± 2.7 (<i>P</i> < 0.01)
<i>Muscle: Sprinters</i>				
FC	19.4 ± 1.6	18.2 ± 1.5 (<i>P</i> < 0.02)	19.4 ± 1.4	21.0 ± 1.7 (<i>P</i> < 0.001)
AC	2.8 ± 0.4	2.9 ± 0.4	2.5 ± 0.4	2.8 ± 0.4
SCAC	3.0 ± 0.4	3.0 ± 0.4	2.7 ± 0.4	2.9 ± 0.4
LCAC	0.38 ± 0.08	0.40 ± 0.08	0.35 ± 0.11	0.40 ± 0.08
TC	22.8 ± 1.8	21.6 ± 1.5 (<i>P</i> < 0.05)	22.5 ± 1.4	24.3 ± 1.4 (<i>P</i> < 0.001)

Values are given as mean ± SD. Our normal values for carnitines in skeletal muscle from 54 nonmyopathic controls are: 19.3 ± 0.8 SD μ mol/g of noncollagenous protein for FC; 2.46 ± 1.28 SD for AC; 2.85 ± 1.5 SD for SCAC; 0.40 ± 0.05 SD for LCAC; 22.0 ± 1.10 SD for TC.

P-values refer to the pretraining concentration.

Table 2. Serum concentration of FC, AC, SCAC, LCAC, and TC (see Table 1) in sprinters and long-distance runners before and after 120 days of training with placebo or L-carnitine administration.

	Placebo				L-Carnitine			
	Baseline		120 days		Baseline		120 days	
	Rest	Exercise	Rest	Exercise	Rest	Exercise	Rest	Exercise
<i>Serum: Long-distance runners</i>								
FC	40 ± 3 *	36 ± 4	38 ± 4	31 ± 5	38 ± 5 *	34 ± 5	39 ± 3	40 ± 3
AC	5.9 ± 1.4 *	10.2 ± 1.3	6.0 ± 1.3 *	11.3 ± 1.3	5.2 ± 1.2 †	11.0 ± 4.2	5.8 ± 0.9 *	20.7 ± 3.1 *
SCAC	6.1 ± 1.3 *	10.6 ± 1.4	6.4 ± 1.3 *	12.0 ± 1.6	5.4 ± 1.3 *	9.9 ± 1.1	6.2 ± 1.0 *	21.7 ± 3.4 *
LCAC	0.9 ± 0.1 †	1.4 ± 0.2	1.0 ± 0.4 †	1.5 ± 0.3	0.8 ± 0.2 †	1.2 ± 0.3	0.8 ± 0.2	0.7 ± 0.1
TC	47 ± 3	48 ± 3	45 ± 6	45 ± 6	45 ± 6	46 ± 6	46 ± 4 *	62 ± 5 *
<i>Serum: Sprinters</i>								
FC	34 ± 4	35 ± 4	37 ± 2	37 ± 2	36 ± 3	35 ± 3	38 ± 2	37 ± 2
AC	3.7 ± 0.3	3.8 ± 0.5	4.0 ± 0.4	3.8 ± 0.3	4.1 ± 0.5	4.2 ± 0.5	4.1 ± 0.6	4.0 ± 0.7
SCAC	3.9 ± 0.9	4.0 ± 0.9	4.2 ± 0.4	4.1 ± 0.3	4.3 ± 0.5	4.4 ± 0.5	4.2 ± 0.6	4.2 ± 0.7
LCAC	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
TC	39 ± 4	40 ± 3	43 ± 2	42 ± 1	41 ± 4	41 ± 3	43 ± 3	42 ± 3

Values are expressed in μ mol/L.

Symbols between columns: "P" values "rest" vs "exercise."

Symbols between rows: "P" values "120 days" vs "baseline."

* = *P* < 0.001; † = *P* < 0.05; ‡ = *P* < 0.01.

exertional decrease of FC ($P < 0.001$), whereas both SCAC ($P < 0.001$) and LCAC ($P < 0.01$) were increased. Since the degree of these opposite changes was of the same order for free and esterified carnitines, TC was not modified. The increase for SCAC was essentially due to a rise in acetylcarnitine, representing up to 90% of the entire SCAC fraction. Training had little effects on these changes (Table 2).

By contrast, the long-distance runners receiving L-carnitine showed a marked increase ($P < 0.001$) of post-exertional TC, AC, and SCAC compared to the resting values. The fall in FC, observed in the placebo group, was not seen in treated athletes. Instead, post-exertional TC values in carnitine-treated long-distance runners were significantly higher ($P < 0.001$) than in placebo-receiving athletes. No significant changes were observed after exercise, training, and L-carnitine administration in the sprinters. Training had little effect in both groups.

Urine (Table 3). In the long-distance runners receiving placebo, physical exercise caused a significant increase of TC excretion, mainly contributed (+100%) by the AC fraction. This phenomenon was amplified by treatment with oral carnitine, which caused increased excretion of the esterified fractions as well as the FC fraction. The latter was obviously a consequence of the treatment itself. Similar, although less marked, effects were ob-

served in the urinary output of carnitines of the sprinters after exercise. Treatment with oral L-carnitine increased the excretion of both free and esterified carnitine. Training influenced neither the relative size of the carnitine fractions nor the total carnitine excretion in the urine.

Morphometry. Type I fibers were predominant in the muscle biopsies of the long-distance runners (type I 49.5 ± 14.5 SD; type IIA 36.3 ± 12.5 SD; type IIB 15 ± 15.8 SD) as opposed to the prevalence of large type II fibers in the sprinters (type I 37.9 ± 12 SD; type IIA 47.7 ± 12.2 SD; 20.6 ± 26.3 SD). These differences did not reach statistical significance and were unchanged by training and/or treatment with L-carnitine.

DISCUSSION

The observation of high carnitine content in long-distance runners, in comparison with the sprinters, was a likely consequence of the higher content of mitochondria in type I fibers, which predominated in the muscles of the long-distance runners. Conversely, the low-carnitine levels observed in sprinters' muscles could be due to the prevalence of type II fibers, in which there are fewer mitochondria.

In both groups of athletes treated with placebo, carnitine content of muscle was decreased

Table 3. Urinary excretion of FC, AC, SCAC, and TC (see Table 1) in sprinters and long-distance runners before and after 120 days of training with placebo or L-carnitine administration.

	Placebo				L-Carnitine			
	Baseline		120 days		Baseline		120 days	
	Rest	Exercise	Rest	Exercise	Rest	Exercise	Rest	Exercise
<i>Urine: Long-distance runners:</i>								
FC	.29 \pm .06	.26 \pm .10	.28 \pm .06	.27 \pm .10	.29 \pm .07	.31 \pm .10	.73 \pm .10 *	.83 \pm .10
AC	.13 \pm .02 *	.37 \pm .02	.18 \pm .04	.40 \pm .09	.15 \pm .003 *	.36 \pm .05	.31 \pm .06 *	.32 \pm .20
SCAC	.24 \pm .04 *	.46 \pm .04	.20 \pm .05	.43 \pm .09	.24 \pm .04	.46 \pm .04	.35 \pm .07 *	.38 \pm .30
LCAC	ND		ND		ND		ND	
TC	.51 \pm .10 †	.72 \pm .10	.48 \pm .10	.71 \pm .10	.53 \pm .10	.76 \pm .10	1.07 \pm .1	2.22 \pm .40
<i>Urine: Sprinters:</i>								
FC	.22 \pm .04	.23 \pm .03	.27 \pm .05	.26 \pm .03	.27 \pm .06	.27 \pm .05	1.07 \pm .2	1.07 \pm .1
AC	.20 \pm .02 †	.22 \pm .02	.23 \pm .01	.27 \pm .05	.23 \pm .02	.24 \pm .03	.32 \pm .03	.32 \pm .03
SCAC	.22 \pm .03 †	.24 \pm .03	.25 \pm .03	.27 \pm .01	.26 \pm .03	.26 \pm .03	.35 \pm .03	.34 \pm .03
LCAC	ND		ND		ND		ND	
TC	.44 \pm .05 *	.47 \pm .05	.52 \pm .08	.53 \pm .08	.52 \pm .09	.53 \pm .08	1.42 \pm .1	1.42 \pm .1

Values are expressed in mmol/g creatinine.

Symbols between columns: "P" values "rest" vs "exercise."

Symbols between rows: "P" values "120 days" vs "baseline."

* = $P < 0.001$; † = $P < 0.05$; ND = not determined.

after training, as compared with basal conditions. The reduction affected the free and total carnitine fractions: these results could lead to a reduced availability of carnitine as a supplier of energetic substrates to mitochondria. Interestingly, these effects were greater in the long-distance runners, whose energetic demand during exercise relies almost exclusively upon the aerobic metabolism of carbohydrates and fatty acids. However, since we did not measure the changes in athletic performance of each group, the functional significance of these biochemical observations must still be established.

In the long-distance runners, but not in the sprinters, the post-exertional blood concentrations of short-chain acylcarnitine and, to a lesser extent, of long-chain acylcarnitine, were increased, while free carnitine was decreased. Skeletal muscle appears as the main, though not the exclusive, source of this increase.

Several factors could contribute to this variation. The increased amount of short-chain acylcarnitine esters in blood (mainly in the form of acetylcarnitine) can be explained by enhanced oxidation of both pyruvate^{10,12} (through the pyruvate dehydrogenase complex) and fatty acids (through the beta-oxidation system) in muscle during the endurance type of exercise performed (3000-meter run). As the production rate of acetylCoA becomes higher than the rate of its utilization through the Krebs cycle, acetylCoA accumulates in mitochondria. CAT provides a clearance mechanism^{12,21} by transforming the excess of acetylCoA which, at high concentrations, may inhibit some enzymatic activities by altering acetylCoA/CoA ratio, into AC; the latter being easily eliminated into the bloodstream. Furthermore, the large mobilization of FFA during prolonged exercise causes rapid accumulation in muscle of LCAC. After exercise, LCAC, which are no longer utilized for oxidative purposes, are returned to the bloodstream. This can explain the moderate post-exertional rise of plasma LCAC.

Altogether, our data indicate that "wasting" of short-chain carnitine esters in the urine occurs from the combined effects of training and prolonged exercise, causing a decrease in the availability of stored muscle carnitine.

An improvement of the endurance capacity by L-carnitine administration in man^{1,16} is still controversial, at least at the biochemical level. However, our data in treated athletes, indicate that oral carnitine supplementation is able to "stabilize" muscle carnitine pools, thus preventing a loss of carnitine

from tissues to the plasma-urine compartments (Table 1).

Interestingly, in long-distance runners and sprinters, serum carnitine at rest remained unchanged after 120 days of oral administration, compared with the "basal" values (Table 2). This is surprising because patients with primary or secondary carnitine deficiencies show increased levels of plasma carnitines under treatment.⁶ In contrast to athletes, however, these subjects have lower-than-normal plasma carnitine levels before treatment. Yet, in the same group of endurance athletes, both total and esterified carnitine clearly increased after exercise. This phenomenon could be due either to a larger carnitine pool in the body, or to its higher utilization for the increased metabolic demands of the trained muscle, or both.

Furthermore, carnitine treatment increased the concentration of acetylcarnitine in plasma and its excretion in the urine. This suggests that a larger carnitine availability can facilitate removal from muscle of the acetylCoA accumulated during exercise, thus maintaining a metabolically favorable acetylCoA/CoA ratio.^{10,12} The post-exertional drop of plasma free carnitine, as well as the rise of plasma LCAC, occurring in the untreated subjects, were no longer observed after treatment with oral carnitine. This result is likely due to the improved utilization of long-chain fatty acids (LCFA) as energetic substrate in muscle. Consequently, the potentially deleterious effects caused by their accumulation are also prevented by oral carnitine administration. In particular, high concentrations of LCFA in mitochondria partially inhibit the activity of adenylate translocase, the enzyme that regulates the ATP-ADP transport from and to the inner compartment of mitochondria.¹¹ Prevention of LCFA inhibition on the translocase activity can improve the efficiency of oxidative phosphorylation coupling and oxygen utilization by mitochondria. This can also explain why endurance athletes, upon L-carnitine loading, have significantly increased values of $\dot{V}O_2 \text{ max}$.¹ This increase, which may be of practical importance in improving tolerance during endurance exercise, has been attributed, in the past, to enhancement of substrate utilization through the Krebs cycle.^{1,10}

Further investigation is necessary to verify whether these observations, still at the biochemical level, can have valuable consequence in improving both the training and performance of endurance athletes.

REFERENCES

1. Angelini C, Vergani L, Costa L, Martinuzzi A, Dunner E, Marescotti C, Nosadini R: Use of L-carnitine in exercise physiology. *Adv Clin Enzymol* 1986;4:103-110.
2. Banker B, Engel AG: Basic reactions of muscle, in Engel AG, Banker B (eds): *Myology*, McGraw-Hill, New York, 1986, pp 845-909.
3. Bremer J: Carnitine metabolism and function. *Physiol Rev* 1983;63:1420-1480.
4. Chalmers RA, Roe RC, Stacey CE, Hoppel CL: Urinary excretion of L-carnitine and acylcarnitines by patients with disorders of organic acid metabolism: evidence for secondary insufficiency of L-carnitine. *Pediat Res* 1984;18:1325-1328.
5. DiDonato S, Rimoldi M, Garavaglia B, Uziel G: Propionylcarnitine excretion in propionic and methylmalonic acidurias: a cause of carnitine deficiency. *Clin Chim Acta* 1984;139:19-21.
6. DiDonato S: Carnitine deficiencies in man, in Gitzelman R, Baerlocher K, Steinmann B (eds): *Carnitine in der Medizin*. Schattauer, Stuttgart, New York, 1987, pp 91-100.
7. Edwards YH, Chase JFA, Edwards MR, Tubbs PK: Carnitine acetyltransferase: the question of multiple forms. *Eur J Biochem* 1974;46:209-215.
8. Engel AG: Quantitative morphological studies of muscle, in Engel AG, Banker B (eds): *Myology*, McGraw-Hill, New York, 1986, pp 1045-1081.
9. Felig P, Wahren J: Fuel homeostasis during exercise. *N Engl J Med* 1975;293:1078-1084.
10. Foster CUL, Harris RC: Formation of acetylcarnitine in muscle of horse during high intensity exercise. *Eur J Appl Physiol* 1987;56:639-642.
11. Haas RH, Stumpf DA: Mitochondrial mechanisms in Reye's like syndromes. *J Natl Reye's Synd Foundat*, 1981;2:61-69.
12. Harris RC, Foster CUL, Hultman E: Acetylcarnitine formation during intense muscular contraction in humans. *J Appl Physiol* 1987;63:440-442.
13. Hoppel CL, Tomec RJ: Carnitine palmitoyltransferase: location of two enzymatic activities in rat liver mitochondria. *J Biol Chem* 1972;247:832-841.
14. Ji LL, Miller RH, Nagle HA, Stratman FW: Amino acid metabolism during exercise in trained rats: the potential role of carnitine in the metabolic fate of branched-chain amino acids. *Metabolism* 1987;36:748-752.
15. Lennon DLF, Stratman FW, Shrager E, Nagle FJ, Madden M, Hanson P, Carter AL: Effects of acute moderate-intensity exercise on carnitine metabolism in man and woman. *J Appl Physiol* 1983;55:489-495.
16. Marconi C, Sassi G, Carpinelli A, Cerretelli P: Effects of L-carnitine and anaerobic performance of endurance athletes. *Eur J Appl Physiol* 1985;54:131-135.
17. Pande SN, Caramacion MN: A simple radioisotopic assay of acetylcarnitine and acetylCoA at picomolar levels. *Anal Biochem* 1981;112:30-38.
18. Rebouche CJ: Carnitine movement across muscle cell membranes. *Biochim Biophys Acta* 1977;471:145-155.
19. Scholte HR, De Jonge PC: Metabolism, function and transport of carnitine in health and disease, in Gitzelman R, Baerlocher K, Steinmann B (eds): "Carnitine in der Medizin". Schattauer, Stuttgart, New York, 1987, pp 22-59.
20. Stumpf DA, Parker WD, Angelini C: Carnitine deficiency, organic acidemias and Reye's syndrome. *Neurology* 1985;35:1041-1045.
21. Uziel G, Garavaglia B, DiDonato S: Carnitine stimulation of pyruvate dehydrogenase complex (PDHC) in isolated human skeletal muscle mitochondria. *Muscle Nerve* 1988;11:720-724.

**RESPIRATORY CHAIN ENZYMES IN MUSCLE OF ENDURANCE ATHLETES:
EFFECT OF L-CARNITINE**

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SUMMARY. The effects of L-carnitine on respiratory chain enzymes in muscle of long distance runners were studied in 14 athletes. These subjects received placebo or L-carnitine (2 g orally b.i.d.) during a 4-week period of training. Athletes receiving L-carnitine showed a significant increase ($p < 0.01$) in the activities of rotenone-sensitive NADH cytochrome c reductase, succinate cytochrome c reductase and cytochrome oxidase. In contrast, succinate dehydrogenase and citrate synthase were unchanged. No significant changes were observed after placebo administration. The levels of both total and free carnitine from athletes receiving placebo were significantly decreased ($p < 0.01$) after treatment. By contrast, total and free carnitine levels were markedly increased ($p < 0.01$) after supplementation with L-carnitine. Our results suggest that L-carnitine induces an increase of the respiratory chain enzyme activities in muscle, probably by mechanisms involving mitochondrial DNA. © 1992 Academic

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Endurance training promotes an increase in the volume and mass of mitochondria relative to other cellular constituents within muscle fibers of the exercised limbs (1). These morphological changes are accompanied by parallel increases in the maximum activities of tricarboxylic acid cycle enzymes of fatty acid transport and oxidation and in part of respiratory chain enzymes (2).

Carnitine performs a crucial role in the energy supply of the muscle during exercise, by controlling the influx of fatty acids into mitochondria (3). In addition, carnitine facilities

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oxidation of pyruvate and branched amino acids (4), and contributes to the protection of cells from these deleterious agents by preventing the accumulation of acyl CoAs (5). During the exercise of long duration, the increased esterification of muscle carnitine reduces the free carnitine pool in muscle, leading to carnitine insufficiency (6).

L-carnitine has been shown to stimulate pyruvate dehydrogenase complex (PDHC) in human skeletal muscle (4). In the same way, the aim of the present study is to asses whether treatment with L-carnitine increases the activities of respiratory chain enzymes in muscle of endurance athletes.

MATERIALS AND METHODS

Subjects and experimental protocol. All the subjects were volunteers and expressed their informed consent to participate in the study. Fourteen well-trained athletes (double blind, parallel groups) were studied. All of them were long-distance runners (LDR), a specialty requiring physical endurance. They have a dietary regimen of 3500 to 4000 Kcal/day, of which proteins represent 13 to 15% of the total caloric intake, and lipids 25% to 30%. Muscle needle biopsies (Vastus lateralis, average net weight: 100-150 mg) were taken at rest to determined basal values of the respiratory chain enzymes and carnitine content. The LDR were then divided at random in two groups: the first group (7 males) was treated with 2 g orally b.i.d. of L-carnitine (Sigma-Tau. Pomezia. Italy) for 28 days; the other group (7 males) received placebo during the same period of time. Carnitine supplementation was suspended 12 hours before muscle sampling. At the same time, the athletes of the two groups started a 4 weeks endurance training program. The weekly training program consisted of running at 40% to 50% VO_2 max for 90 minutes/day for 5 days, and at 70% to 80% of the VO_2 max for 60 minutes for the other 2 days, corresponding to 130-140 Km/wk. At the end of the training period (28 days) a second biopsy was performed (at rest) and carnitine content, as well as enzyme activities, were re-examined.

Carnitine assay. Free carnitine (FC), short-chain acylcarnitine (SCAC), long-chain acylcarnitine (LCAC) and total carnitine were measured in muscle homogenates as described by Di Donato et al (7).

Enzyme assays. Muscle biopsies were homogenized in 15 volumes of 0.15 M KCl, 50 mM Tris-HCl pH 7.4 in all glass homogenizers and mitochondrial enzymes were measured in supernatants after centrifugation at 800xg for 10 minutes. Describe spectrophotometric assays (8) were used to measure succinate cytochrome c reductase (complex II+III), rotenone-sensitive NADH-cytochrome c reductase (complex I+III), succinate dehydrogenase (complex II), citrate synthase and cytochrome c oxidase. The latter was determined by monitoring the decrease in absorbance at 550 nm of reduced cytochrome c. Reduced cytochrome c was freshly prepared before each experiment by adding to a 1% solution in 10 mM K-Phosphate buffer (pH 7.0). Protein concentration was determined by Lowry method (9).

Statistical analysis. Statistical analysis was performed by one way analysis of variance and Student's test for paired and unpaired comparison.

RESULTS

Respiratory chain enzymes (Table 1): The basal levels of all respiratory enzymes as well as citrate synthase in muscle of LDR were markedly increased compared to normal age and sex matched control values. The pretreatment activities of all the enzymes in athletes receiving L-carnitine or placebo were similar. LDR receiving L-carnitine showed a significantly increase ($p < 0.01$) of the activities of NADH cytochrome c reductase, succinate cytochrome c reductase and cytochrome c oxidase after treatment, compared to both the pretreatment and the posttreatment receiving placebo levels. In contrast, succinate dehydrogenase and citrate synthase activities remained unchanged. No significant changes were observed after placebo administration.

Carnitine (Table 2): In basal conditions, muscle free and total carnitine content in muscle of LDR were both markedly increased compared to normal controls. No significant differences in the pretreatment amounts of TC, FC, SCAC and LCAC were observed between carnitine-treated athletes and placebo-receiving athletes. In LDR receiving placebo the levels of both TC and FC were significantly lower ($p < 0.01$) after treatment than before treatment, whereas SCAC was unchanged and LCAC was significantly increased ($p < 0.01$). By contrast, TC and FC levels were significantly higher ($p < 0.01$) after treatment than before treatment in LDR supplemented with L-carnitine. However, SCAC and LCAC remained unchanged. In addition, the difference between carnitine-treated athletes and placebo-receiving athletes after treatment was markedly significant ($p < 0.01$) for FC and TC.

DISCUSSION

In agreement with previous reports (1,2), the values of all respiratory chain enzymes as well as the citrate synthase basal

Table 1. Succinate dehydrogenase (SDH, complex II), rotenone-sensitive NADH-cytochrome c reductase (NADH cyt c red, complex I+III), succinate cytochrome c reductase (succ cyt c red, complex II+III), cytochrome c oxidase (Cox, complex IV) and citrate synthase (CS) in muscle of endurance athletes

	n	SDH	NADH cyt c red	succ cyt c red	Cox	CS
Controls	25	10.3±3.6	11.7±4.0	7.5±3.1	52.8±16.7	120±38.5
Long distance runners	14	15.2±4.3*	24.3±6.95*	13.1±4.8*	129.6±26.6*	309.8±68.1*
Placebo	7					
Before treatment		14.9±3.8	25.5±7.7	13.5±5.6	131.7±27.7	307.3±68.2
After treatment		14.8±2.2	27.2±8.3	14.4±6.7	130.8±31.5	304.9±67.9
L-carnitine	7					
Before treatment		15.6±4.7	23.2±6.2	12.7±4.1	127.5±25.4	312.3±73.2
After treatment		15.8±5.7	41.1±9.8*	22.6±7.2*	198.8±22.7*	299.8±66.5*

Activities expressed as nmol of substrate utilized. $\text{min}^{-1} \cdot \text{mg protein}^{-1}$ (mean±SD).

* Significant differences vs controls ($p < 0.01$; unpaired t test).

+ Significant differences vs both pretreatment levels and receiving placebo posttreatment levels ($p < 0.01$; one way variance analysis).

Table 2. Muscle free carnitine (FC), short chain acylcarnitine (SCAC), long chain acylcarnitine (LCAC) and total carnitine (TC) in endurance athletes

	n	FC	SCAC	LCAC	TC
Controls	30	19.3±0.8	2.86±1.50	0.40±0.05	22±1.10
Long distance runners	14	27.5±3.3*	2.75±0.70	0.36±0.075	30.7±3.7*
Placebo	7				
Before treatment		27.3±3.1	2.71±0.71	0.34±0.07	30.4±3.5
After treatment		24.1±3.2†	2.76±0.80	0.48±0.09†	27.3±3.7†
L-carnitine	7				
Before treatment		27.8±3.5	2.8±0.7	0.38±0.08	30.98±3.8
After treatment		31.7±2.9§	2.7±0.8	0.37±0.09	34.8±3.4§

Values are expressed in $\mu\text{mol. g}^{-1}$ of noncollagenous protein (mean \pm SD).

* significant differences vs controls ($p < 0.01$; unpaired t test).

† significant differences vs pretreatment levels ($p < 0.01$; paired t test).

§ significant differences vs both pretreatment and posttreatment receiving placebo levels ($p < 0.01$; one way variance analysis).

values (table 1) were markedly increased in muscle of long-distance runners (LDR). The mechanisms leading to these effects are still unclear, but have been extensively reviewed elsewhere (10).

Our results show that treatment with L-carnitine causes a significantly increase of the activities of NADH cytochrome c reductase, succinate cytochrome c reductase and cytochrome c oxidase in muscle of LDR. The mechanism whereby L-carnitine induces this shift in the respiratory chain enzyme pattern is unknown yet, but mtDNA is likely to be involved (i.e. by stimulation of either mtDNA replication or transcription, or both of them), because of mtDNA codes for several polypeptides of the complexes I, III and IV (11). In contrast, the activities of succinate dehydrogenase, a mitochondrial respiratory chain enzyme encoded by nuclear DNA, and citrate synthase, a enzyme of the mitochondrial matrix, remained at pretreatment levels. Some recent findings indicate that acetyl-L-carnitine treatment is able to stimulate mitochondrial transcription under altered metabolic conditions, such as ageing (12) and hypothyroidism (13). In these conditions, the correct structure and function of mitochondrial membranes appear to be altered (14,15), but acetyl-L-carnitine normalizes the induced alterations. In the same way, in endurance exercise, membrane destabilizing agents, such as acyl CoAs can accumulate giving rise to reduced free carnitine pool in muscle (6,16).

Our results indicate that oral carnitine supplementation is able to "stabilize" muscle carnitine pool, thus preventing a loss of carnitine from tissues to plasma-urine compartments (6). A larger carnitine availability might normalize the alteration in the lipid composition of human muscle mitochondria. The latter is particularly relevant for mtDNA transcription influencing factors such as optimal mitochondrial concentration of ATP and cations (17), import into mitochondria of RNA polymerase and other nuclear DNA encoded proteins required for mtRNA synthesis (18) and processing (19).

However, mitochondrial enzymes levels remained unchanged after treatment in athletes receiving placebo. These data indicate that the training program had little influence, if any, on mitochondrial function.

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REFERENCES

1. Saltin B., and Gollnick P.D. (1983) In *Handbook of Physiology. Skeletal muscle.* pp 555-631. American Physiological Society, Bethesda.
2. Holleszy J.O., and Coyle E.F. (1984) *J. Appl. Physiol.* **56**, 831-838.
3. Bremer J. (1983) *Physiol. Rev.* **63**, 1420-1480.
4. Uziel G., Garavaglia B., Di Donato S. (1988) *Muscle Nerve* **11**, 720-724.
5. Stumpf D.A., Parker W.D., Angelini C. (1985) *Neurology* **35**, 1041-1045.
6. Arenas J., Ricoy J.R., Encinas A.R., Pola P., D'Iddio S., Zeviani M., Di Donato S., and Corsi M. (1991) *Muscle Nerve* **14**, 598-604.
7. Di Donato S., Rimoldi M., Garavaglia B., Uziel G. (1984) *Clin. Chim. Acta* **139**, 13-21.
8. Di Mauro S., Servidei S., Zeviani M., Di Rocco M., De Vivo D., Di Donato S., Uziel G., Berry G., Hoganson K., Johnsen G., and Johnson P.C. (1987) *Ann. Neurol.* **22**, 498-506.
9. Lowry O.H., Rosenbrough N.J., Farr A.L., and Randall R.J. (1951) *J. Biol. Chem.* **193**, 265-275.
10. Sanders Williams R. (1986) In *Biochemical aspects of physical exercise* (Benzyl L et al; eds) Elsevier, pp 171-180. Amsterdam.
11. Attardi G. (1984) *Trends Biochem. Sci.* **6**, 86-103.
12. Gadaleta M.N., Petruzzella V., Renis M., Fracasso F., and Cantatore P. (1990) *Eur. J. Biochem.* **187**, 501-506.
13. Gadaleta M.N., Petruzzella V., Fracasso F., Fernández Silva P., and Cantatore P. (1990) *FEBS Lett.* **277**, 191-193.
14. Hansford R.G., and Castro F. (1982) *Mech. Ageing Dev.* **19**, 191-201.
15. Hafner R.P., Leake M.J., and Brond M.D. (1989) *FEBS Lett.* **248**, 175-178.

16. Angelini C., Vergani L., Costa L., Martinuzzi A., Dunner E., Marescotti C., and Nosadini R. (1986) *Adv. Clin. Enzymol.* **4**, 103-110.
17. Gaines G., Rossi C., and Attardi G. (1987) *J. Biol. Chem.* **262**, 1907-1915.
18. Fisher R.P., Topper J.N., and Clayton D.A. (1987) *Cell* **50**, 247-258.
19. Topper J.N., and Clayton D.A. (1990) *Nucl. Acid. Res.* **18**, 789-793.

[11] Staelens, B., van Tol, A., Chan, L., Will, H., Verhoeven, G. and Auwerx, J. (1990) *Endocrinology* 127, 1144-1152.

[12] Salter, A.M., Hayashi, R., Al-Seeni, M., Brown, N.F., Bruce, J., Sorensen, O., Atkinson, B.A., Middleton, B., Bleasdale, R.C. and Bindley, D.N. (1991) *Biochem. J.* 276, 825-832.

[13] Day, R., Gebhard, R.L., Schwartz, H.L., Strait, K.A., Duane, W.C., Stone, B.G. and Oppenheimer, J.H. (1989) *Endocrinology* 125, 459-468.

[14] Kremers, E.P., Doctor, R., Bernard, B., Visser, T. and Hennemann, G. (1982) *FEBS Lett.* 140, 229-233.

[15] Sogol, P.B., Hersman, J.M., Reed, A.W. and Dillmann, W.H. (1983) *Endocrinology* 113, 1464-1468.

[16] Aanderud, S., Sundsford, J. and Aarbakke, J. (1984) *Endocrinol. Ogy* 115, 1605-1608.

[17] Nees, G.C., Pendleton, L.C., Li, Y.C. and Chiang, J.Y.L. (1990) *Biochem. Biophys. Res. Commun.* 172, 1150-1156.

[18] Bakker, O., van Beeren, M.H. and Wiersinga, W.M.J. (1993) *Endocrinol. Invest.* 16 (suppl. 2-6), 95.



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Effects of L-carnitine on the pyruvate dehydrogenase complex and carnitine palmitoyl transferase activities in muscle of endurance athletes

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Abstract

The effects of L-carnitine on the pyruvate dehydrogenase (PDH) complex and carnitine palmitoyl transferase (CPT) were studied in muscle of 16 long-distance runners (LDR). These subjects received placebo or L-carnitine (2 g orally) during a 4-week period of training. Athletes receiving L-carnitine showed a dramatic increase ($P < 0.001$) in the PDH complex activities. By contrast, the levels of CPT, both 1 and 2, were unchanged. No significant changes were observed after placebo administration. We previously reported (Huertas, R. et al., *Biochem. Biophys. Res. Commun.* 188 (1992) 102-107) that L-carnitine induces an increase in the activities of complexes I, III and IV of the respiratory chain in muscle of LDR. Taken together, our data suggest that the improvement in (maximal oxygen consumption) $V_{O_2 \text{max}}$ observed in LDR after L-carnitine administration is based on these biochemical findings.

Key words: Endurance; Carnitine palmitoyl transferase; Pyruvate dehydrogenase complex; Mitochondria

1. Introduction

The energetic demand during endurance exercise relies almost exclusively upon the aerobic metabolism of carbohydrates and fatty acids. The pyruvate oxidation rate is controlled by pyruvate dehydrogenase complex activity. The PDH complex is composed of three catalytic enzymes (PDH, lipoamide transacylase, and lipoamide dehydrogenase), and two regulatory enzymes (PDH kinase and PDH phosphatase) [1].

The long-chain fatty acid (LCFA) oxidation rate is controlled by CPT activity. CPT has two functional locations within the mitochondrion [2]: CPT 1, is located on the inner surface of the outer mitochondrial membrane and CPT 2 is situated on the inner surface of the inner mitochondrial membrane.

The oxidation of LCFA is almost completely dependent on carnitine [2]. Moreover, carnitine and carnitine acetyl transferase (CAT) are known to control the acetyl CoA/CoA ratio and therefore pyruvate oxidation [3].

Recently, we documented [4] a marked increase in activities of complexes I, III and IV of the respiratory chain in muscle of endurance athletes receiving L-carnitine. The aim of the present work is to assess how

endurance exercise modifies PDH and CPT activities in long-distance runners with and without carnitine supplementation.

2. Materials and methods

2.1. Experimental protocol

All the subjects were volunteers and expressed their informed consent to participate in the study. Sixteen well-trained male athletes (double blind, parallel groups) were studied. All of them were LDR, a speciality requiring physical endurance. Age was 28.3 ± 7.1 years (mean \pm S.D.), body weight was 67 ± 5.1 kg (mean \pm S.D.), Controls ($n = 22$) were all sedentary age-matched males. Body weight was 69.2 ± 6.7 (mean \pm S.D.).

Both, controls and athletes, had a dietary regimen of 3,500 to 4,000 kcal/day, of which proteins represented 13% to 15% of the total caloric intake, and lipids 25% to 30%. Muscle needle biopsies (Vastus lateralis, average net weight: 100-150 mg) were taken at rest to determine basal values of the PDH complex and CPT. The LDR were then divided at random in two groups: the first group was treated with 2 g orally of L-carnitine (Sigma-Tau) for 28 days; the other group received placebo during the same period of time.

Carnitine supplementation was suspended 12 h before muscle sampling. At the same time, the athletes of the two groups started a 4 weeks endurance training program. The weekly training program consisted of running below the anaerobic threshold, 40% to 50% of the maximal oxygen consumption ($V_{O_2 \text{max}}$ for 90 min/day for 5 days, and at the anaerobic threshold, 70% to 80% of the $V_{O_2 \text{max}}$ for 60 min for the other 2 days, corresponding to 130-140 km per week. At the end of the training period, 28 days, a second biopsy was performed (at rest) and the PDH complex and CPT activities, were re-examined. All biopsies were immediately frozen and stored in liquid nitrogen until analysis, which was done at the end of the protocol.

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2.2. Biochemical assays

Muscle biopsies were homogenized in 15 volumes of 0.15 M KCl, 50 mM Tris-HCl pH 7.4 in all-glass homogenizers. CPT was measured in total muscle homogenates by the forward reaction (CPT 1) [5], and by the backward reaction (CPT 2) [6]. PDH complex activity was determined in total muscle homogenates by a radiochemical method as described [7]. Non-collagen protein (NCP) was measured by the method of Lilienthal et al. [8].

2.3. Statistical analysis

Statistical analysis was performed by one-way analysis of variance and Student's *t*-test for paired and unpaired comparison.

3. Results

3.1. PDH complex activities (Table 1)

Basal levels of the PDH complex in muscle of LDR were significantly increased ($P < 0.05$) compared to age- and sex-matched control values. Pretreatment activities in athletes receiving L-carnitine or placebo were similar. LDR receiving L-carnitine showed a dramatic increase ($P < 0.001$) in the activities of PDH complex after treatment compared to pretreatment levels. By contrast, in LDR receiving placebo, no significant changes were observed after placebo administration. After treatment, LDR receiving L-carnitine had activities markedly higher ($P < 0.001$) than LDR receiving placebo.

3.2. CPT activities (Table 1)

Under basal conditions, CPT activities, both 1 and 2, in muscle of LDR were markedly increased ($P < 0.001$ and $P < 0.01$, respectively) compared to age- and sex-matched control levels. Pretreatment activities in athletes receiving L-carnitine or placebo were similar. Both in athletes receiving placebo and in those receiving carnitine pretreatment levels did not differ significantly from post-treatment levels.

3.3. Carnitine and citrate synthase activities

Data on carnitine content and citrate synthase (CS) activities in muscle of these LDR have been recently reported elsewhere [4]. (Table 2).

Under basal conditions, muscle free and total carnitine content in muscle of LDR were both markedly increased compared to normal controls. No significant differences in the pretreatment amounts of total carnitine (TC), free carnitine (FC), short chain acylcarnitine (SCAC) and long chain acylcarnitine (LCAC) were observed between carnitine-treated athletes and placebo-receiving athletes. In LDR receiving placebo the levels of both TC and FC were significantly lower ($P < 0.01$) after treatment than before treatment, whereas SCAC was unchanged and LCAC was significantly increased ($P < 0.01$). By contrast, TC and FC levels were significantly higher ($P < 0.01$) after treatment than before treatment in LDR supplemented with L-carnitine. However, SCAC and LCAC remained unchanged. In addition, the difference between carnitine-treated athletes and placebo-receiving

athletes after treatment was markedly significant ($P < 0.01$) for FC and TC.

The basal levels of CS in muscle of LDR were markedly increased compared to normal age- and sex-matched control values. CS of LDR receiving L-carnitine remained unchanged. No significant changes were observed after placebo administration in CS activity.

4. Discussion

We found that the activities of the PDH complex were significantly increased in muscle of long-distance runners (Table 1). The reasons for these exercise-induced biochemical effects are unknown yet. Possible mechanisms involved have been reviewed elsewhere [9].

Our results show that LDR had a marked increase in CPT 1 levels, and a less marked, but very significant, increase in CPT 2 activities. These findings suggest that during prolonged exercise both CPT 1 and 2, could play a central role in controlling the flux of fatty acids into the oxidation pathway on human skeletal muscle. Exercise-induced increase of CPT 2 activity has been previously shown in rat skeletal muscle [10]. However, to our knowledge, this is the first report that demonstrates an increase of CPT 1 and 2 in muscle of LDR during endurance exercise.

Our data indicate that treatment with L-carnitine dramatically increased the activities of PDH complex in muscle of LDR. Moreover, oral carnitine supplementation is able to 'stabilize' muscle carnitine pool. By contrast, the activities of CPT remained at pretreatment levels. The catalytic efficiency of PDH complex is regulated by a phosphorylation-dephosphorylation mechanism of the α -subunit [11]. PDH kinase, the inhibiting and phosphorylating compound is stimulated by a high acetyl-CoA/CoA ratio. During endurance exercise, py-

Table 2						
	<i>n</i>	FC	SCAC	LCAC	TC	CS
Controls	30	19.3 ± 0.8	2.86 ± 1.50	0.40 ± 0.05	22 ± 1.10	120 ± 38.5
	14	27.5 ± 3.3*	2.75 ± 0.70	0.36 ± 0.075	30.7 ± 3.7*	309.8 ± 68.1*
	7	27.3 ± 3.1	2.71 ± 0.71	0.34 ± 0.07	30.4 ± 3.5	307.3 ± 68.2
Placebo						
	Before treatment	24.1 ± 3.2*	2.76 ± 0.80	0.48 ± 0.09*	27.3 ± 3.7*	304.9 ± 67.9
	After treatment	7	27.8 ± 3.5	2.8 ± 0.7	0.38 ± 0.08	30.98 ± 3.8
L-Carnitine						
	Before treatment	31.7 ± 2.9*	2.7 ± 0.8	0.37 ± 0.09	34.8 ± 3.4*	299.8 ± 66.5*
	After treatment					

Carnitine: Values are expressed in $\mu\text{mol} \cdot \text{g}^{-1}$ of noncollagenous protein (mean ± S.D.). *Significant differences vs. controls ($P < 0.01$; unpaired *t*-test).

Citrate synthase: Activities are expressed in $\text{nmol of substrate utilized min}^{-1} \cdot \text{mg NCP}^{-1}$ (mean ± S.D.). *Significant differences vs. controls ($P < 0.01$); unpaired *t*-test. *Significant differences vs. both pretreatment levels and receiving placebo posttreatment levels ($P < 0.001$; one-way variance analysis).

ruvate is oxidized at high rates. As the production rate of acetyl-CoA becomes higher than the rate of its utilization through the Krebs cycle, acetyl-CoA accumulates in mitochondria. CAT, together with L-carnitine, provide a clearance mechanism by transforming the excess of acetyl-CoA into acetyl/carnitine. A larger carnitine availability in muscle, therefore, can stimulate PDH complex activity by forming larger amounts of acetyl/carnitine from pyruvate-derived acetyl-CoA. In agreement with our results, Uziel et al. [7] in an 'in vitro' study on isolated human muscle mitochondria, reported that at pyruvate concentrations above 0.25 mM, only carnitine concentrations greater than 0.1 mM stimulate PDH complex activity.

Endurance athletes upon L-carnitine loading increase the values of $V_{\text{O}_2 \text{max}}$, which may be of practical importance in improving tolerance during exercise [12]. Several lines of evidence, at the biochemical level, seem to support such a physiological response; first, L-carnitine induces an increase of the respiratory chain enzyme activities in muscle [4]; second, L-carnitine stimulates PDH complex activity in muscle; and third, L-carnitine loading prevents the accumulation of LCFA [13], which are potentially deleterious inhibitors of adenylylate translocase in muscle. However, we can not rule out the possibility that L-carnitine treatment may affect further factors, i.e. β -oxidation enzymes.

We conclude that in muscle from endurance athletes, when pyruvate is oxidized at high rates, L-carnitine and CAT are able to regulate PDH activity by maintaining a favorable acetyl-CoA/CoA ratio. This conclusion is consistent with our data previously reported [13], which showed that in LDR L-carnitine treatment increased the

References

- [1] Linn, T.C., Pettit, F.H. and Reed, C.J. (1969) Proc. Natl. Acad. Sci. USA 62, 234-241.
- [2] Brenner, J. (1990) Clin. Biochem. 23, 297-301.
- [3] Chalmers, R.A., Roe, C.R., Tracey, B.M., Stacey, T., Hoppe, C.L. and Millington, D.S. (1983) Biochem. Soc. Trans. 11, 724-725.
- [4] Huertas, R., Campos, Y., Diaz, E., Esteban, J., Vebrietti, L., Montanari, G., D'Addio, S., Corsi, M. and Arenas, J. (1992) Biochem. Biophys. Res. Commun. 188, 102-107.
- [5] Zierz, S. and Engel, A. (1985) Eur. J. Biochem. 149, 207-214.
- [6] Di Manlio, S. and Melis-Di Manlio, P. (1973) Science 182, 929-931.
- [7] Uziel, G., Garavaglia, B. and Di Donato, S. (1988) Muscle Nerve 11, 720-724.
- [8] Lilienthal, J.L., Zierer, K.L. and Folk, B.P. (1950) J. Biol. Chem. 183, 501-508.
- [9] Sanders Williams, R. (1986) In: Biochemical Aspects of Physical Exercise (Bentz, L., et al., Eds.), pp. 171-180. Elsevier, Amsterdam.
- [10] Guzman, M. and Castro, J. (1988) Biochim. Biophys. Acta 963, 562-565.
- [11] Cate, R.L. and Roche, T.B. (1979) J. Biol. Chem. 254, 1659-1665.
- [12] Angelini, C., Vergani, L., Costa, L., Martinuzzi, A., Dunner, E., Maresco, C. and Nosadini, R. (1986) Adv. Clin. Enzymol. 4, 103-110.
- [13] Arenas, J., Ricoy, J.R., Encinas, A.R., Pela, P., Di Daddio, S., Zavial, M., Di Donato, S. and Corsi, M. (1991) Muscle Nerve 14, 598-604.

Activities are expressed in $\mu\text{mol of substrate utilized min}^{-1} \cdot \text{mg NCP}^{-1}$ (mean ± S.D.). *Significant differences vs. controls ($P < 0.05$); unpaired *t*-test. *Significant differences vs. controls ($P < 0.01$); unpaired *t*-test. *Significant differences vs. both pretreatment levels and receiving placebo post-treatment levels ($P < 0.001$; one-way variance analysis).